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1. Studies were continued to perfect a standard model system for evaluating the recovery of viable microorganisms from the interior of plastic (methymethacrylate-lucite). A considerable die-away during the process of polymerization occurred which agreed with earlier observations. This reduction in the viable spore population (Bacillus subtilis var. niger) was approximately one log in all cases. After polymerization, the rate of die-away was lower and appeared to be governed by both time and temperature of storage.

   Experiments were performed to determine the number of spores destroyed by the grinding technique (blender-mill) and the number which remained embedded in the plastic after grinding. Discs of lucite, seeded with spores and weighing approximately 0.5 g, were surface-sterilized. The actual number of viable spores per gram of plastic was determined by dissolving the sample in acetone and assaying the suspension by means of a solvent-resistant membrane filter incubated on trypticase soy agar (TSA).

   Some samples were ground for 1 or 5 minutes in the blender-mill and the resultant suspension assayed by pour plates and standard membrane filters.

   Other samples were ground for 1 or 5 minutes and the suspension passed through standard membrane filters which subsequently were dissolved in acetone. This suspension, which included the spores freed from the plastic particles by dissolution, was assayed with a solvent-resistant membrane filter. The general procedure is outlined in Figure 1.

   The blender-mill method recovered approximately 2 logs fewer viable spores than the control method (dissolution). Both methods showed that a die-away occurred during storage at 25 C (Figure 2). After polymerization, no further die-away was observed when the plastic was stored at -4 C (Figure 3). No significant loss of viable spores was noted in plastic discs kept at - 4 C for 216 hours and assayed by the dissolution method. Grinding for 1 minute did not appear to be sufficient for release of all viable spores because a one-log increase occurred following dissolution of the ground particles (Figure 4). Grinding for 5 minutes, however, appeared to release all viable spores present in the plastic because no increase was observed after dissolution (Figure 5). When the temperature was varied, no significant die-away was noted if discs were stored at -4 C, but a slight die-away did occur at room temperature (Figures 4 and 5). Fewer viable spores were recovered from dissolved 5-min. than from 1-min.grindings. The longer grinding period probably released more spores but some may have been destroyed by the combination of grinding and production of internal heat.

2. Studies concerning the enumeration of microbial contaminants on surfaces were continued. Tests were performed to determine the effect of temperature on the recovery of B. subtilis var. niger spores from surfaces by ultrasonication.

Three experiments, two with stainless steel strips and one with smooth glass, comparing cold (4 C) with room-temperature (25 C) peptone water, revealed that in all cases the percent recovery of spores in cold peptone water was significantly higher than in warm peptone water. The average number of spores recovered also was greater in two of three cases. In other studies the temperature of both the peptone water and the bath solution were varied. These experiments were conducted on both stainless steel and smooth glass. Results agreed with the preliminary findings. With peptone water at 4 C and the tank solution at 25 C, both the average number of spores recovered and the average percent recovery were higher than with any other combination of factors tested. In most cases the differences were considered significant (Table 1).

When mechanical agitation was used as the recovery method, no significant differences in recovery were observed when warm or cold peptone water was used as recovery medium.

Although the question of why more microorganisms were recovered when peptone water at 4 C rather than 25 C was used cannot be explained at this time, there is a practical application for this phenomenon. In assessing the levels of microbial contamination on space hardware or on stainless steel strips exposed to spacecraft assembly and test areas, assays are made for bacterial spores. This is done by heating the rinse water from the sample at a temperature high enough to kill all of the vegetative microorganisms but low enough to allow the spores to survive. However, when a large number of samples is being assayed, they may of necessity remain at room temperature for 30 to 40 minutes before being heat-shocked. During this time it is probable that some of the spores begin to germinate and lose their heat resistance. Since this could result in a reduction of colony-forming units, the accuracy of the assay system might be affected. The germination of spores can be retarded significantly by cold temperatures. Since higher recoveries are obtained using cold rather than room-temperature peptone water, the use of cold peptone water would not only improve the overall efficiency of the assay system, but also increase the sensitivity for detecting and enumerating bacterial spores.

3. Several experiments were performed to evaluate the efficiency and precision of the swab-rinse technique which is presently being used to assess levels of surface contaminants on lunar spacecraft. Two sets of stainless steel strips which had been exposed to natural airborne contamination for 3 and 4 weeks, respectively, were used. Half of each set was assayed in the usual manner by means of ultrasonication to determine the mean number of microorganisms per strip. The remaining strips were swabbed and the number of microorganisms removed from the strip, as well as the number recovered from the swab using ultrasonication, were determined. Four similar experiments were performed using strips artificially contaminated with an aerosol of <u>B. subtilis</u> var. <u>niger</u> spores. For these tests both cotton and calcium alginate swabs were evaluated. A solution of sodium hexametaphosphate in peptone water was used for dissolving the alginate swabs. The results showed that the removal of microorganisms from stainless steel surfaces by swabs was a more consistent factor than the recovery from the swab (Tables 2, 3, and 4 ).

Tests involving five individuals using a standard swab-rinse technique indicated that there was a small but statistically significant difference between the percentage of spores removed from stainless steel surfaces by certain individuals. However, no consistent or significant difference between individuals was evident based on percent recovery (Table 5). The use of calcium alginate swabs did not improve removal or recovery. In addition, it appeared that agitation with a vortex-mixer prior to ultrasonication increased recovery of spores from cotton swabs. (Table 3).

4. Studies on the recovery of sublethally-injured microorganisms were continued. Some investigators have noted that significantly higher colony counts of spores exposed to ethylene oxide (ETO) occurred when culture plates were incubated for 50 rather than the usual 2 days. To investigate this further, tubes and stainless steel strips were inoculated with a suspension of B. subtilis var. niger spores. After drying under vacuum, the samples were exposed to 600 mg/L of ETO for 1 and 2 hours. Trypticase soy broth (TSB) was added to each of the tubes and 3 decimal dilutions were made with TSB. The tubes were incubated at 32 C and observed for the presence of visible growth at intervals up to 35 days. The inoculated strips were treated in the same manner except that the suspensions were plated with TSA and colony counts made. The results showed there was an increase in colony counts and in the number of tubes turning positive during the 2- and 5-day incubation interval. Between 5 and 35 days no significant increases were observed (Tables 6 and 7).

As reported earlier, extremely long "tails" were observed in the destructive curves of B. subtilis var. niger spores exposed to 120 C dry heat. It was determined that this was due to a technique-induced artifact. During the drying process under vacuum, the spores, which were inoculated onto the bottom of sterile test tubes, apparently migrated to the top of the tube. Since this area of the tube was not immersed in the oil bath, there was an extreme temperature differential which allowed a small portion of the population to survive. No tails were observed when the neck of the tube was cut at a point which had been below the level of the oil bath or when small stainless steel strips were inoculated and then placed in the tubes.

Three tests, each consisting of six replicates and six intervals, were made at 125 C using an ethanol suspension of B. subtilis var. niger spores on stainless steel. The mean D value was 30.5 minutes (Figure 6). Eventually 10 to 15 typical spacecraft surfaces will be tested to determine their effect on the dry-heat resistance of spores.

In an attempt to standardize procedures used to determine D values of spores exposed to dry heat and in accordance with a request from the NASA, suspensions of B. subtilis var. niger spores, stainless steel surfaces, media and a protocol were sent to six research groups involved in similar dry-heat studies.

5. At Cape Kennedy monitoring of microbial contamination was continued in Hangars S, AE, and AO, and in the Surveyor sterilization and assembly laboratory and fuel loading room. Similar studies were initiated in the Lunar Orbiter camera room and the Biosatellite clean room (Tables 8, 9, and 10).

   Further tests were performed to determine the level of microbial contamination on the surfaces of two Lunar Orbiter spacecraft (Table 11).

   As reported earlier, very few anaerobic spores have been detected on the surfaces of the Surveyor and Lunar Orbiter spacecraft by the swab-rinse technique. To determine if this was due to the absence of anaerobic spores or failure of the technique to detect them, two trays containing stainless steel strips were exposed to the intramural air of the east bay of the Surveyor sterilization and assembly laboratory for 3 and 6 weeks, respectively. Half of each set of strips was assayed by the swab-rinse test and the other half by the standard rinse technique of placing each strip in 50 ml of 1% peptone water, ultrasonicating and plating. The results showed that the rinse test recovered more microorganisms and anaerobic spores than the swab-rinse test (Table 12). This was attributed to the fact that some microorganisms removed by swabs were not eluted during the rinsing process. There was no evidence that suggested the assay technique was detrimental to the contaminants. Consequently, the low numbers of anaerobic spores detected on certain spacecraft were probably due to the combination of low contamination levels and failure to free all microorganisms from the cotton.

6. Work was initiated on the development of the NASA manual, "Procedures for Evaluation of Microbiological Control Laboratories and Spacecraft Control Facilities."

7. The construction of the bioclean laboratory support facility was completed and a contract was let for the construction of the vertical laminar flow clean room.

TABLE 1. EFFECTS OF VARYING TEMPERATURES OF PEPTONE WATER AND ULTRASONIC TANK SOLUTION ON RECOVERY OF SPORES OF *BACILLUS SUBTILIS* VAR. *NIGER* FROM SURFACES.

| Surface | Temperature of peptone water | Temperature of tank solution | Avg. no. of spores recovered+ | Probability* factor | Avg. percent recovery | Probability* factor |
|---|---|---|---|---|---|---|
| Stainless steel | 4 C | 25 C | 416 | -- | 92.1 | -- |
| Stainless steel | 4 C | 4 C | 318 | $<0.001$ | 79.7 | $<0.01$ |
| Stainless steel | 25 C | 25 C | 394 | $>0.20$ | 89.5 | $>0.20$ |
| Stainless steel | 25 C | 4 C | 316 | $<0.001$ | 82.0 | $<0.001$ |
| Smooth glass | 4 C | 25 C | 389 | -- | 94.9 | -- |
| Smooth glass | 4 C | 4 C | 354 | $>0.05$ | 85.6 | $<0.01$ |
| Smooth glass | 25 C | 25 C | 350 | $<0.01$ | 88.6 | $<0.02$ |
| Smooth glass | 25 C | 4 C | 354 | $<0.05$ | 89.2 | $>0.05$ |

* Probability factors are for comparison with peptone water at 4 C and ultrasonic tank solution at 25 C.

+ Mean of 15 samples.

TABLE 2. REMOVAL AND RECOVERY OF NATURAL MICROBIAL CONTAMINATION FROM STAINLESS STEEL SURFACES USING COTTON SWABS.

| Test No. | Procedure | No. of strips | Percent removal | Percent recovery | Coefficient of variation for percent recovery |
|---|---|---|---|---|---|
| 1 | Cotton swab; swab ultrasonicated | 21 (Test)<br>21 (Controls) | 87 | 97 | 60% |
| 2 | Cotton swab; swab ultrasonicated | 24 (Test)<br>24 (Controls) | 86 | 56 | 60% |

TABLE 3. REMOVAL AND RECOVERY OF BACILLUS SUBTILIS VAR. NIGER SPORES FROM STAINLESS STEEL SURFACES USING COTTON SWABS.

| Test no. | Procedure | No. of strips | Percent removal | Percent recovery | Coefficient of variation for percent recovery |
|---|---|---|---|---|---|
| 1 | Cotton swab; swab ultrasonicated | 20 (Test)<br>20 (Control) | 82 | 36 | 20% |
| 2 | Cotton swab; swab mixed* and ultrasonicated | 60 (Test)<br>23 (Control) | 86 | 89 | 12% |

* Each swab was agitated with a vortex-mixer prior to ultrasonication.

TABLE 4. REMOVAL AND RECOVERY OF _BACILLUS_ _SUBTILIS_ VAR. _NIGER_ SPORES FROM STAINLESS STEEL SURFACES USING CALCIUM ALGINATE SWABS.

| Test no. | Procedure | No. of strips | Percent removal | Percent recovery | Coefficient of variation for percent recovery |
|---|---|---|---|---|---|
| 1 | Calcium alginate swab; swab dissolved in 6 ml of solution* | 21 (Test)<br>21 (Control) | 76 | 29 | 37% |
| 2 | Calcium alginate swab; swab dissolved in 25 ml of solution | 20 (Test)<br>20 (Control) | 70 | 36 | 31% |

*1 ml of 10% sodium hexametaphosphate in 5 ml of 1% peptone water. All suspensions were ultrasonicated for 12 minutes prior to plating.

TABLE 5. REMOVAL AND RECOVERY OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES FROM STAINLESS STEEL SURFACES USING COTTON SWABS.

| Individual performing test | No. of strips* | Percent removal | Coefficient of variation for percent removal | Percent recovery | Coefficient of variation for percent recovery |
|---|---|---|---|---|---|
| A | 12 | 86 | 6% | 92 | 17% |
| B | 12 | 89 | 4% | 89 | 20% |
| C | 12 | 83 | 6% | 89 | 14% |
| D | 12 | 86 | 3% | 87 | 17% |
| E | 12 | 87 | 5% | 85 | 23% |

* 23 strips were used as controls.

TABLE 6. EFFECT OF EXTENDED INCUBATION ON SPORES OF BACILLUS SUBTILIS VAR. NIGER EXPOSED TO 600 MG/L OF ETHYLENE OXIDE AND RECOVERED IN TRYPTICASE SOY AGAR.

| Hours of exposure to ethylene oxide | Dilution | Mean number[a] of visible colonies per plate after incubation at 32 C | | | | | |
|---|---|---|---|---|---|---|---|
| | | No. of days 2 | No. of days 5 | No. of days 7 | No. of days 14 | No. of days 28 | No. of days 35 |
| 1 | $10^{-1}$ | 31.9 | 39.3 | 39.9 | 39.9 | 39.9 | 39.9 |
| | $10^{-2}$ | 2.7 | 4.1 | 4.2 | 4.2 | 4.2 | 4.2 |
| 2 | none | 0 | 0 | 0 | 0 | 0 | 0 |
| Controls: not exposed to ethylene oxide[b] | $10^{-5}$ | 86.5 | 86.9 | 86.8 | Sp.[c] | | |
| | $10^{-6}$ | 8.1 | 8.2 | 8.2 | 8.2 | 8.2 | 8.2 |

[a] Each value is the mean colony count from 25 samples.

[b] Each value is the mean colony count from 6 samples.

[c] Colonies coalesced and could not be counted accurately.

TABLE 7. EFFECT OF EXTENDED INCUBATION ON SPORES OF BACILLUS SUBTILIS VAR. NIGER EXPOSED TO 600 MG/L OF ETHYLENE OXIDE AND RECOVERED IN TRYPTICASE SOY BROTH.*

| Hours of exposure to ethylene oxide | Dilution | No. of tubes | Number of tubes showing visible growth after incubation at 32 C | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | No. of days 2 | No. of days 5 | No. of days 7 | No. of days 14 | No. of days 21 | No. of days 28 | No. of days 35 |
| 1 | none | 25 | 25 | | | | | | |
| | $10^{-1}$ | 25 | 25 | | | | | | |
| | $10^{-2}$ | 25 | 11 | 25 | | | | | |
| | $10^{-3}$ | 25 | 0 | 17 | 17 | 19 | 19 | 19 | 19 |
| 2 | none | 25 | 0 | 1 | 2 | 2 | 2 | 2 | 2 |
| | $10^{-1}$ | 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $10^{-2}$ | 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $10^{-3}$ | 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* The trypticase soy broth consisted of 15 g trypticase, 5 g phytone, and 5 g NaCl, in 1 liter of distilled water. The pH was 7.

TABLE 8. ACCUMULATION OF MICROORGANISMS ON STAINLESS STEEL STRIPS EXPOSED TO THE INTRAMURAL ENVIRONMENT OF THE LUNAR ORBITER CAMERA ROOM FOR 11 WEEKS.

| Weeks of exposure | Aerobes No./$ft^2$ | Aerobic spores No./$ft^2$ | Anaerobes No./$ft^2$ | Anaerobic spores No./$ft^2$ |
|---|---|---|---|---|
| 1 | 23,040 | 238 | 1,080 | 302 |
| 2 | 1,022 | 122 | 122 | 58 |
| 3 | 3,658 | 122 | 238 | 122 |
| 4 | 540 | 58 | 58 | 0 |
| 5 | 302 | 58 | 58 | 0 |
| 7 | 958 | 0 | 302 | 122 |
| 8 | 2,160 | 0 | 1,022 | 122 |
| 10 | 25,798 | 302 | 1,138 | 0 |
| 11 | 1,699 | 238 | 0 | 0 |

TABLE 9. ACCUMULATION OF MICROORGANISMS ON STAINLESS STEEL STRIPS EXPOSED TO THE ENVIRONMENT OF THE BIOSATELLITE CLEAN ROOM. SITE 1.

| Weeks of exposure | Aerobic vegetative No./$ft^2$ | Anaerobic vegetative No./$ft^2$ | Aerobic spores No./$ft^2$ | Anaerobic spores No./$ft^2$ |
|---|---|---|---|---|
| 2 | 8,460 | 1,138 | 122 | 0 |
| 4 | 216 | 72 | 58 | 0 |
| 5 | 122 | 0 | 122 | 0 |
| 6 | 1,138 | 180 | 238 | 58 |
| 7 | 540 | 540 | 58 | 0 |
| 8 | 302 | 0 | 122 | 0 |
| 9 | 778 | 122 | 360 | 58 |
| 10 | 238 | 302 | 58 | 0 |

TABLE 10. ACCUMULATION OF MICROORGANISMS ON STAINLESS STEEL STRIPS EXPOSED TO THE ENVIRONMENT OF THE BIOSATELLITE CLEAN ROOM. SITE 2.

| Weeks of exposure | Aerobic vegetative No./$ft^2$ | Anaerobic vegetative No./$ft^2$ | Aerobic spores No./$ft^2$ | Anaerobic spores No./$ft^2$ |
|---|---|---|---|---|
| 2 | 6,242 | 778 | 0 | 0 |
| 4 | 418 | 122 | 0 | 0 |
| 5 | 0 | 0 | 58 | 0 |
| 6 | 1,800 | 0 | 0 | 0 |
| 7 | 238 | 78 | 0 | 0 |
| 8 | 1,080 | 122 | 482 | 0 |
| 9 | 540 | 0 | 58 | 0 |
| 10 | 122 | 0 | 238 | 0 |

TABLE 11. LEVEL OF MICROBIAL CONTAMINATION ON THE SURFACE OF THE LUNAR ORBITER AS DETERMINED BY THE SWAB-RINSE TECHNIQUE.

| Spacecraft | Area sampled ($in.^2$) | Date | Mesophilic microorganisms | | | |
|---|---|---|---|---|---|---|
| | | | Aerobes | Anaerobes | Aerobic spores | Anaerobic spores |
| #5 | 57.4 | 7-29-66 | 50 | 15 | 0 | 0 |
| #5 | 57.4 | 10-17-66 | 25 | 15 | 0 | 5 |
| #5 | 57.4 | 10-27-66 | 165 | 55 | 10 | 20 |
| #6 | 57.4 | 10-17-66 | 35 | 15 | 10 | 0 |

TABLE 12. COMPARISON BETWEEN THE SWAB-RINSE AND RINSE TECHNIQUES[a] FOR THE RECOVERY OF MICROORGANISMS FROM STAINLESS STEEL STRIPS.

| Type of mesophilic microorganisms recovered | Recovery method | No. of samples | Mean colony count | Probability factor |
|---|---|---|---|---|
| Aerobic vegetative | Swab-rinse[b] | 30 | 1,932 | $>0.4$ |
| | Rinse[c] | 30 | 2,882 | |
| Anaerobic vegetative | Swab-rinse | 30 | 323 | $>0.1$ |
| | Rinse | 30 | 115 | |
| Aerobic spores | Swab-rinse | 30 | 13 | $<0.05$ |
| | Rinse | 30 | 44 | |
| Aerobic spores | Swab-rinse | 30 | 131 | $\geq 0.3$ |
| | Rinse | 30 | 241 | |
| Anaerobic spores | Swab-rinse | 30 | 2 | $\leq 0.01$ |
| | Rinse | 30 | 17 | |
| Anaerobic spores | Swab-rinse | 30 | 41 | $>0.3$ |
| | Rinse | 30 | 160 | |

[a] Stainless steel strips were exposed to the intramural environment of areas used for the test and assembly of the Surveyor spacecraft.

[b] Each strip was swabbed with a cotton swab which in turn was placed in a tube of 1% peptone water, ultrasonicated, and the resultant suspension plated.

[c] Strips were assayed in the standard fashion by placing each in 50 ml of 1% peptone water, ultrasonicating, and plating the suspension.

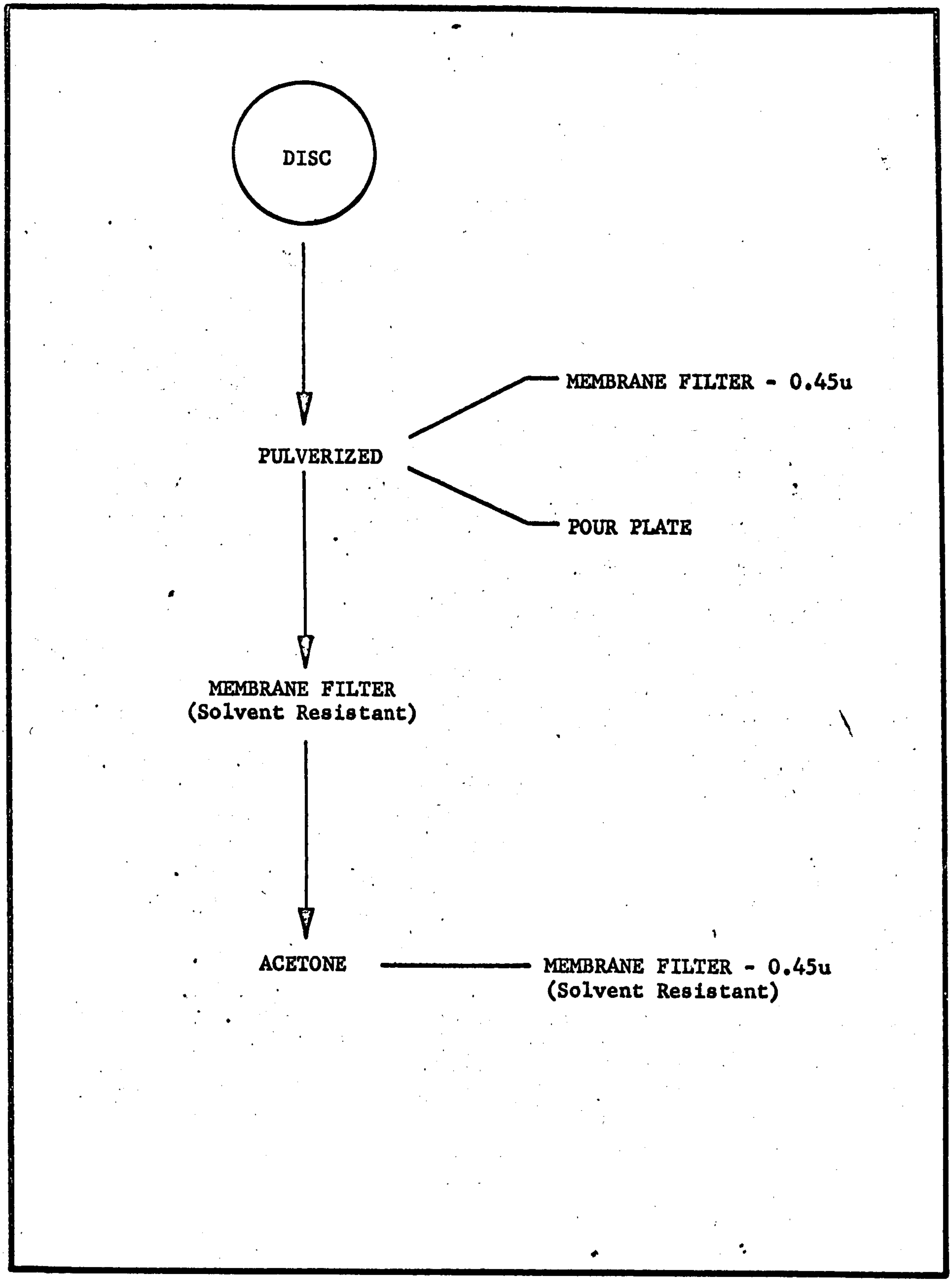


Fig. 1. General procedure used to assay discs of lucite seeded with Bacillus subtilis var. niger spores.

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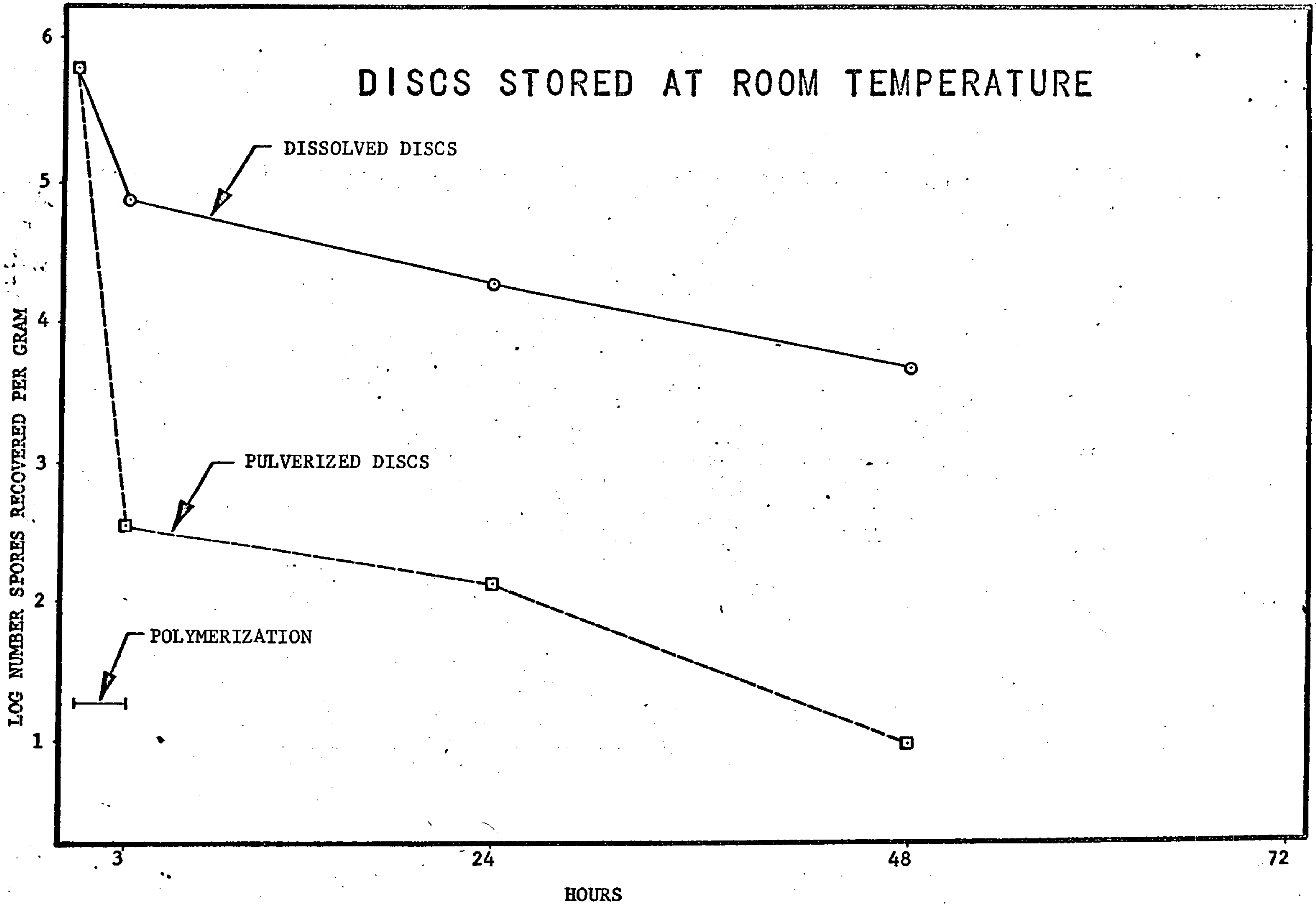


Fig.2. Effect of polymerization and storage on the survival of B. subtilis var. niger spores in lucite.

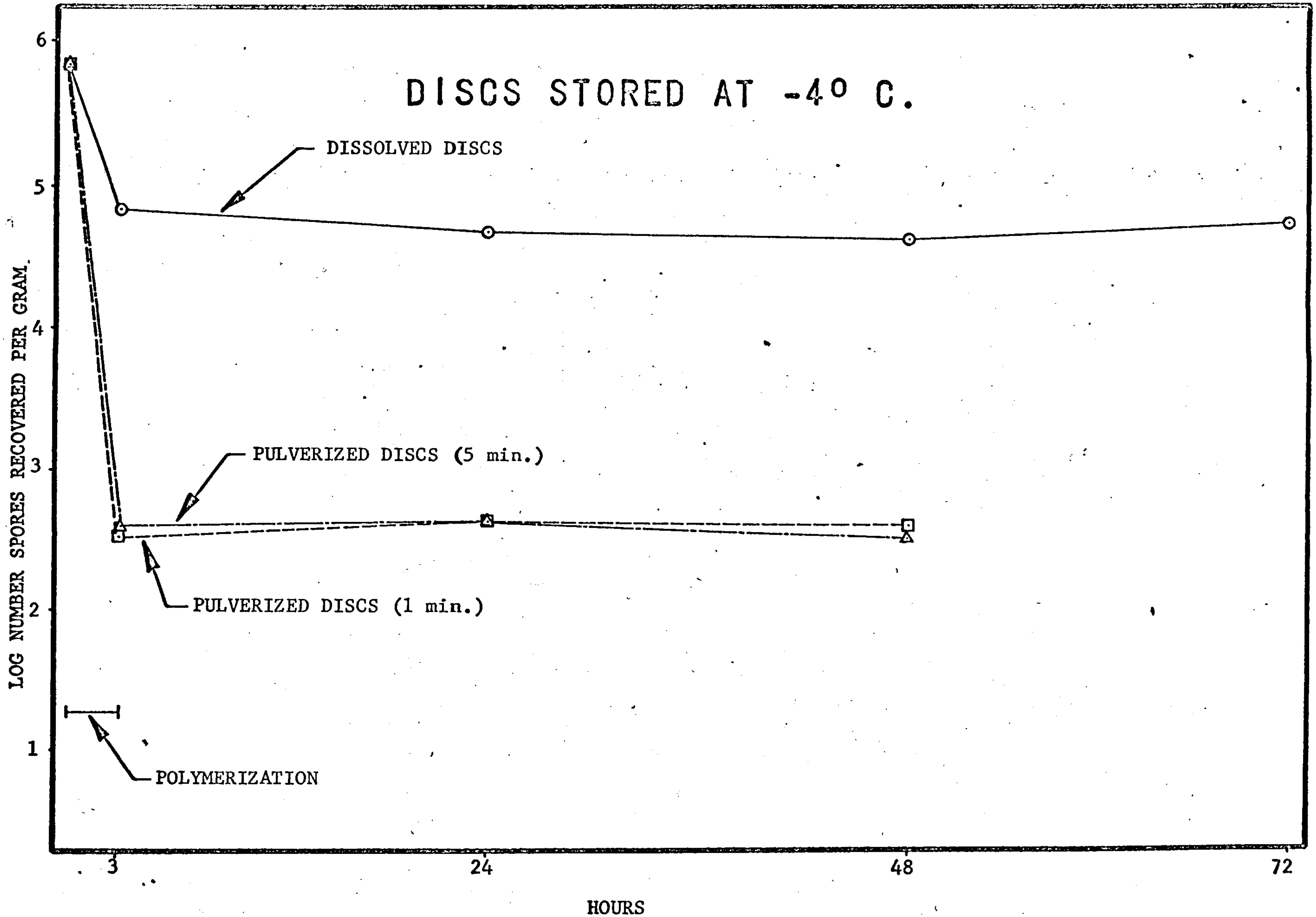


Fig. 3. Survival of B. subtilis var. niger spores in lucite stored at -4 C.

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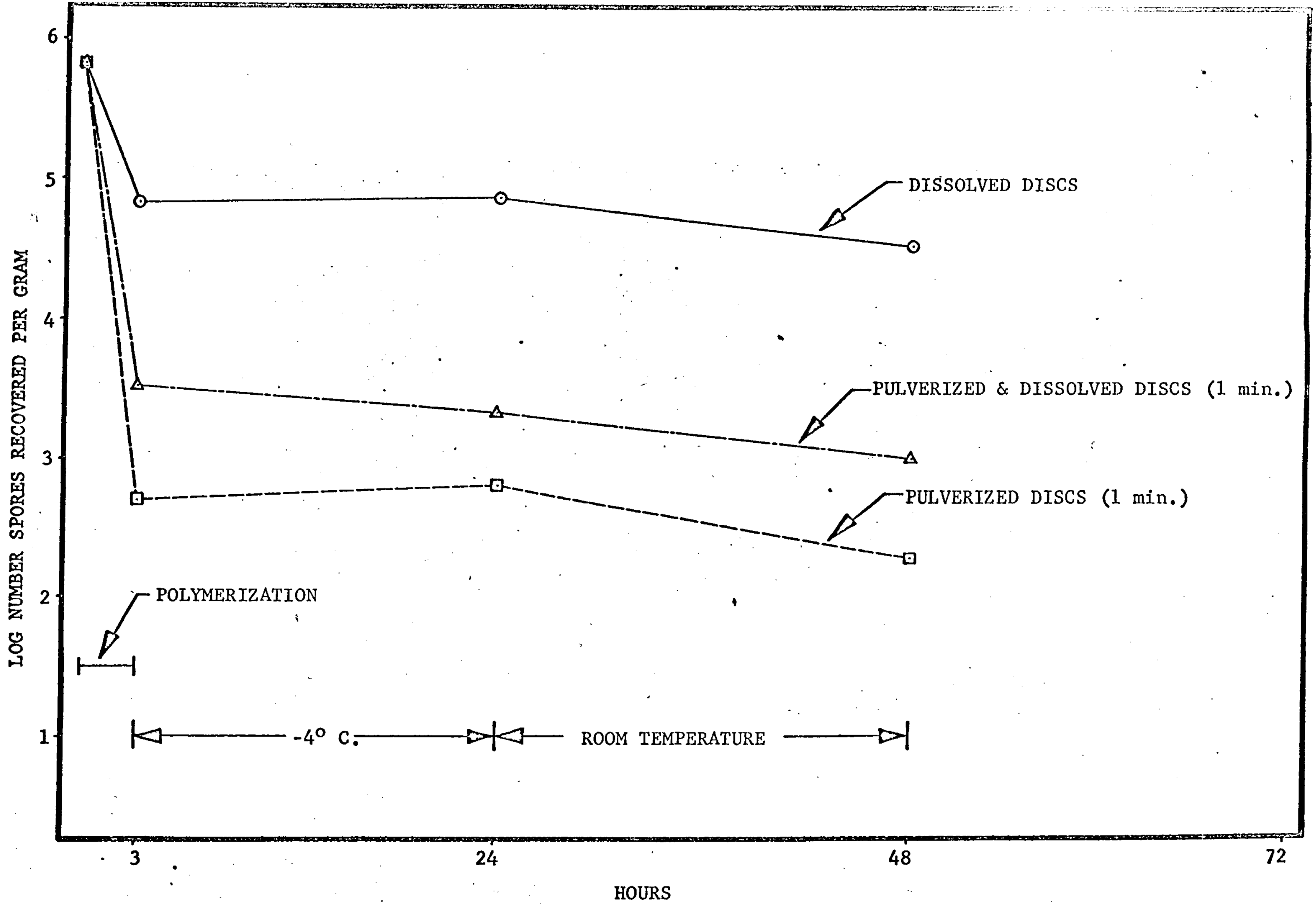


Fig. 4. Recovery of B. subtilis var. niger spores from lucite stored at two temperatures and pulverized for 1 minute.

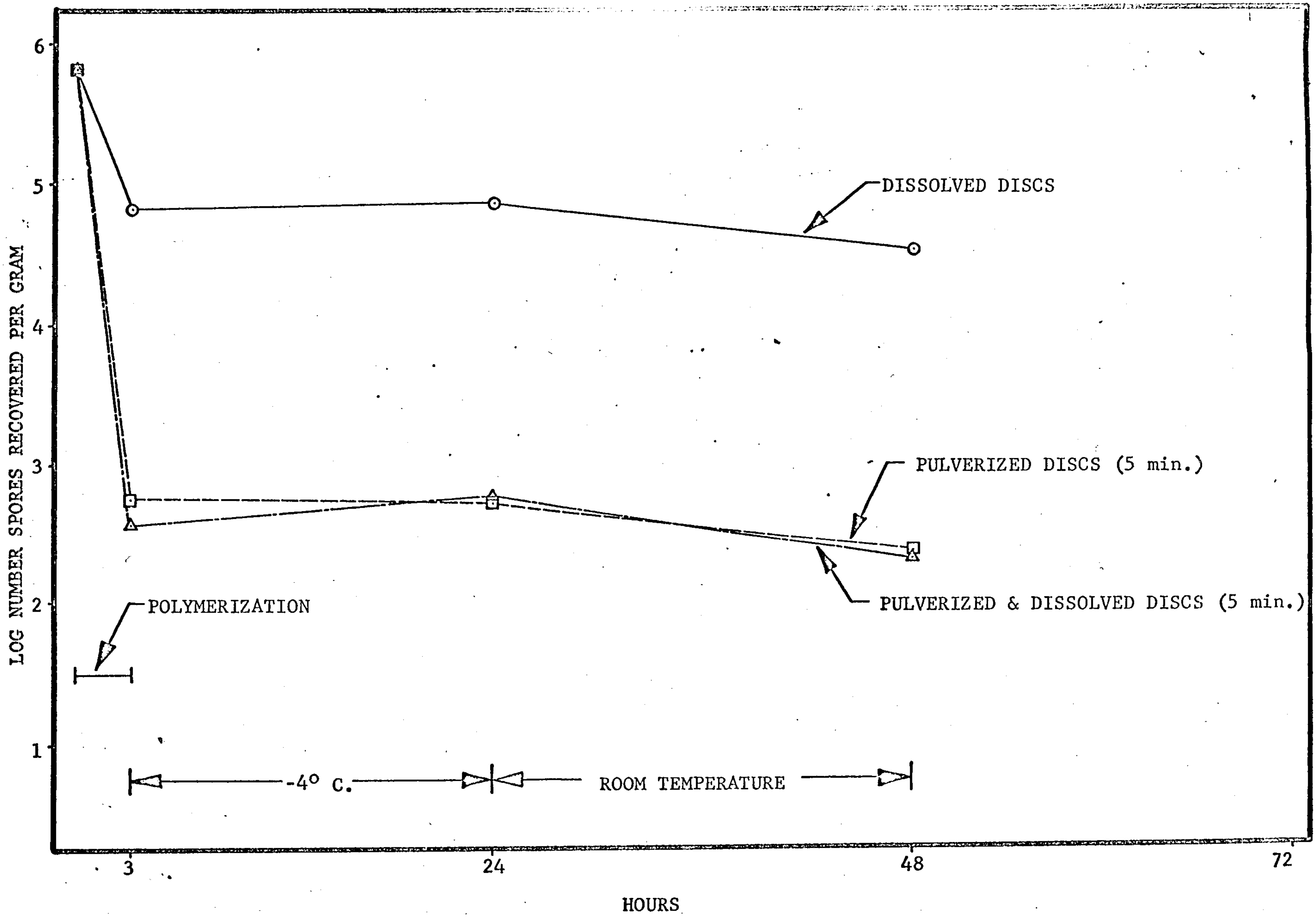


Fig. 5. Recovery of B. subtilis var. niger spores from lucite stored at two temperatures and pulverized for 5 minutes.

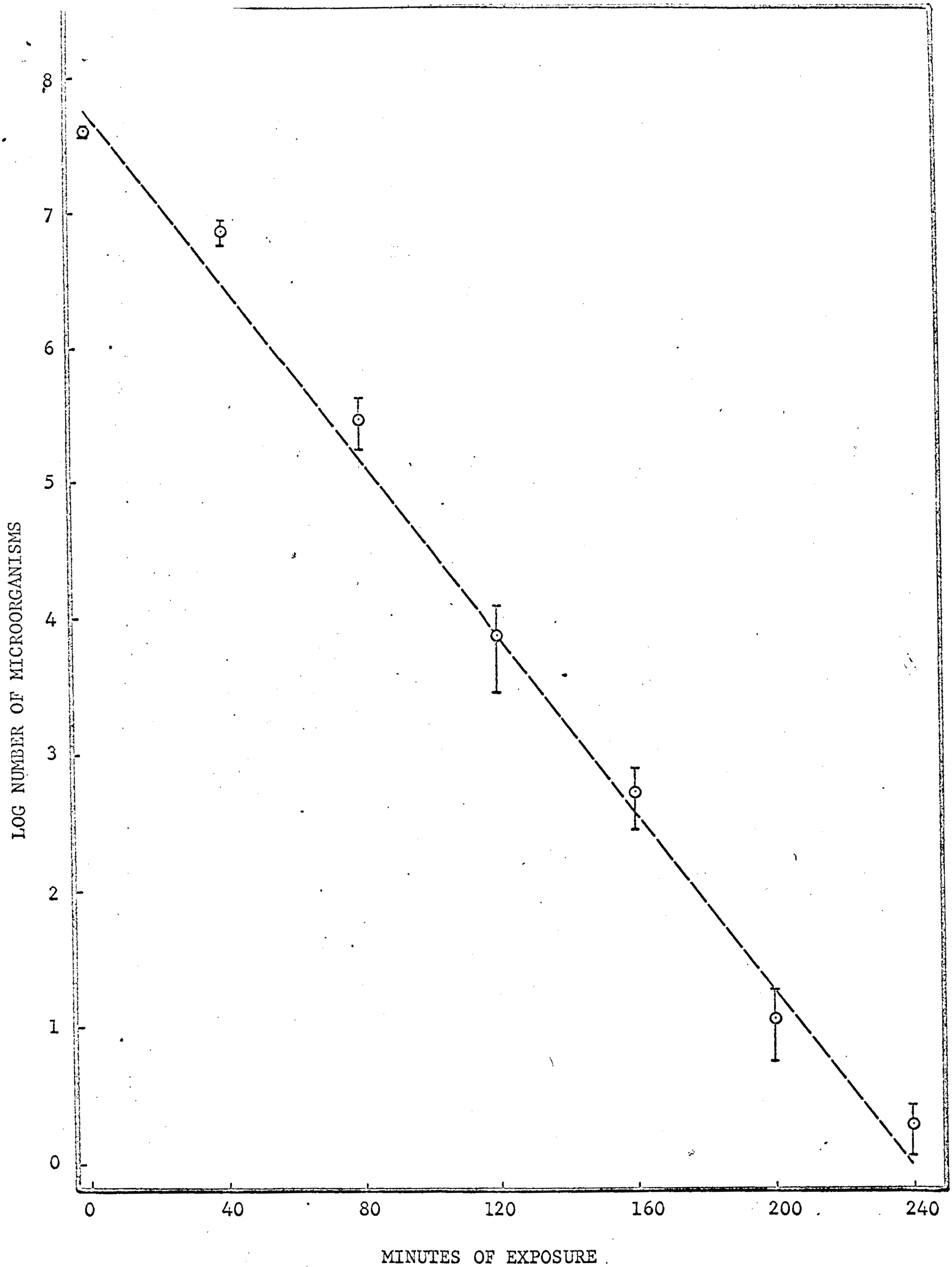


Fig. 6. Dry heat inactivation of <u>Bacillus</u> <u>subtilis</u> var. <u>niger</u> spores at 125 C. Each point is the mean of 3 experiments ± one standard error.